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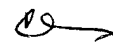
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## Introduction

Breast cancer has been the leading cause of death among non-smoking women and thus has been the focus of intensive research. For the last decade or so, many researchers have concentrated on understanding the molecular basis of breast cancer. Since the epithelial cells of the breast are regulated by a variety of hormones and growth factors, it appears that abnormal hormonal milieu might be one of the critical factors in the development of breast cancer. EGF (epidermal growth factor) is a growth-stimulating factors and act as an autocrine and paracrine growth factor. The expression of both EGF and is positively regulated by estrogen and progesterone receptors [1].

The EGF receptor is a 170 kDa transmembrane glycoprotein, which belong to the tyrosine kinase receptor family. The role of the EGF receptor in breast cancer has been studied in great detail over the last decade [2], but it still remains under debate. The expression of EGF receptors on normal breast epithelial cells is low and elevated expression of EGF receptors occurs in about 40% of primary breast cancers. There is a clear reverse relationship between EGF receptor expression and estrogen receptor expression, as well as between the expression of EGF receptor and progesterone receptor [3]. High levels of EGF receptor are associated with poor tumor differentiation, high tumor grade, aneuploidy and high rate of cell proliferation [4]. There is still no consensus, however, on the significance of EGF receptor in breast cancer prognosis or its correlation with the relapse-free survival and overall survival [5,6]. In node-positive patients, the EGF receptor appears to be a good prognostic marker, but in the node-negative patients, the EGF receptor does not appear to have a prognostic value [7,8].

The development of the transgenic mouse and gene knockout techniques provides an exceptional opportunity to elucidate the role of the EGF receptor signal pathway in mammary gland cancer [9]. Three groups have generated null mutations of the EGF receptor in mouse by homologous recombination in embryonic stem (ES) cells. The phenotype of the null mutation in mouse turned out to be different in different genetic backgrounds. However, no female mice live long enough to develop mature mammary gland [10-12]. Thus, the null mutation of the EGF receptor in mouse does not provide us with a model to study the role of the EGF receptor in mammary cancer. Therefore, we need to generate a mutant mouse lacking EGF receptor specifically in the mammary gland. Recent success of cre-loxP mediated gene knockout technique [13], which allows one to change the gene of interest in a tissue-specific manner, provides us with the tool to generate mice lacking EGF receptor only in the mammary gland. This mutant mouse will be our model to study the role of the EGF receptor in mammary cancer. We will evaluate the effect of the EGF receptor on the incidence of mammary tumors induced by chemical carcinogenesis. Analysis of this mutant mouse should reveal the role of the EGF receptor in the etiology and progression of breast cancer and will indicate the significance of the EGF receptor as a therapeutic target.

## Body

### **1) Construction of the targeting vector for EGF receptor gene. (Task 1 in statement of work)**

The targeting vector was finished at the first year as show in figure 1.

### **2) Cloning of the promoter region of WAP (white acid protein) gene and construction of WAP-*cre*. (Task 2 in statement of work)**

We have cloned the promoter region of WAP and made the construct containing 3.5 kb WAP promoter and *cre* gene. After we made the construct, Jackson Lab has obtained the license from DuPont to sale transgenic mouse containing *cre* gene, including WAP-*cre* mouse, which is the same one that we plan to generate. Therefore, in stead of generating our own strain of mouse, we will purchase WAP-*cre* mouse from Jackson Lab when we need it, but Jackson Lab has not received them yet. Now, Dr. Wagner is willing to provide us the WAP-Cre mice.

### **3) Electroporation of ES cells with EGF receptor construct. (Task 3 in statement of work)**

We have introduced the EGF receptor targeting vector we constructed into ES cells by electroporation. After ten days of G418 selection, we have isolated about 500 colonies. Each individual clone was expanded and the DNA from each clone is isolated for Southern blot analysis. The probe we used for Southern blot analysis is a ~500 bp fragment 5'-end outside targeting construct (see figure1). After analyzing more than 400 individual clones, we identified 11 positive ones. We expanded the ES cells from those 11 positive clones for more DNA to confirm those ES cells had gone through the correct DNA recombination by Southern blot analysis (figure 3a). We also use the *neo* gene as a probe to determine a single integration.

### **4) Electroporation of ES cells with the *cre* gene to remove the *neo* gene cassette. (Task 3 in statement of work)**

One of positive clones was selected to remove the *neo* gene cassette. The ES cells was electroporated with a plasmid containing *cre* gene under the control of CMV promoter. About 20 G418 negative clones were picked up. However, Southern blot analysis revealed that all of them were type II deletion (figure 2), and we expected to get 50% type I deletion and 50% type II deletion. We further tried several times with the same result. After attending the "Mouse Molecular Genetics" meeting, we found out some other labs have the same problem as we have. In order to solve the problem, we took several approaches. We used a different promoter, such as PGK, to express *cre* gene in ES cells and lowered the amount of the *cre* gene construct for eletroporation. After several trials, we were able to resolve the problem. Now we have both type I and type II deletion of ES cells. From the meeting, we have also learned that we do not have to generate

both type I and type II deletion for our study to understand the role of EGF receptor in mammary gland tumor. Recently, many labs have shown transgenic mice containing *cre* recombinase is sufficient to delete the exon of gene of interest to more than 80%. Apparently, it becomes not necessary for us to generate type II deletion mice for our studies and we will only need to generate type I homozygous mice that carrying two copies of loxP sites flanking exon 1 of EGF receptor. As a result, it will save a lot of time to generate our mouse model for our studies.

#### **5) Generating mouse containing loxP sites flanking exon 1 of EGF receptor gene. (Task 5 and 6 in statement of work)**

To generate mouse containing loxP sites flanking exon 1 of EGF receptor, we injected ES cells with type I deletion to generate chimeric mice. However, none of the chimeric animals are able to give rise to germ-line transmission. Recent studies from Westphal's Lab and others have shown that transgenic mice carrying a *cre* gene under the control of EIIa promoter are able to delete sequence at the zygote stage as well as to delete the *neo* gene cassette at a high percentage [14]. Therefore, we also injected the ES cells that contain the targeting vector (figure 1) to generate chimeric mice. Those chimeric animals were able to give rise to germ-line transmission. The heterozygous mice that contain the targeting vector were crossed with a transgenic mice carrying a *cre* gene under the control of EIIa promoter, which was kindly provided by Dr. Westphal. In order to use PCR to genotype the mice, we design two sets of oligo primers that flanking the sequence of two loxP sites between 100 bp to 200 bp apart (see figure 1, primer 1 / primer 2 and primer 3 / primer 4), so that we are able to use PCR to determine whether the mice is carrying a specific loxP site in the genome as well as carrying a deletion of the *neo* gene cassette. For primer 1 / primer 2, the mutant allele has insertion of a loxP site of ~50 bp. Therefore, the PCR product will show two bands when both the wild type and mutant alleles exist (figure 3b). The upper one is the mutant allele and the lower one is the wild type allele. For primer 3 / primer 4, only when the *neo* gene cassette is deleted, the PCR product will show two bands. The lower band is the wild type allele, and the upper one is the mutant allele that contains one loxP site without the *neo* gene cassette. In the case that the *neo* gene cassette is not deleted, the PCR product will only show one band (the wild type band) because the other band would have been more than 2.5 kb, and it usually will not show a band due to over the limit of length Taq polymerase can produce. We have used this method to genotype our mice from their DNA very efficiently (figure 3b and 3c). Crossing with EIIa-Cre mice, more than 70% (10/14) of the *neo* gene cassette (type I deletion, see figure 2) from the mice that are carrying the targeting vector are deleted. The percentage of deletion of the *neo* gene cassette was very high. Those mice with deletion of the *neo* gene cassette are further crossing with the wild type mice to mate out the EIIa-Cre gene to generate type I deletion mice, and are ready to mate with WAP-Cre mice.

## Summary (key research accomplishments)

- Targeting vector for cre-loxP mediated EGF receptor gene knockout.
- ES cells with EGF receptor targeting construct.
- Chimeric mice that gave rise to germ-line transmission.
- Mice carrying the targeting construct.
- Mice carrying two loxP sites flanking exon 1 of EGF receptor gene with the deletion of the *neo* gene cassette by mating with EIIa-Cre mice.
- Mice carrying the genotype of EGFR<sup>flox/+</sup> by mating out the EIIa-Cre gene from the mouse genome.

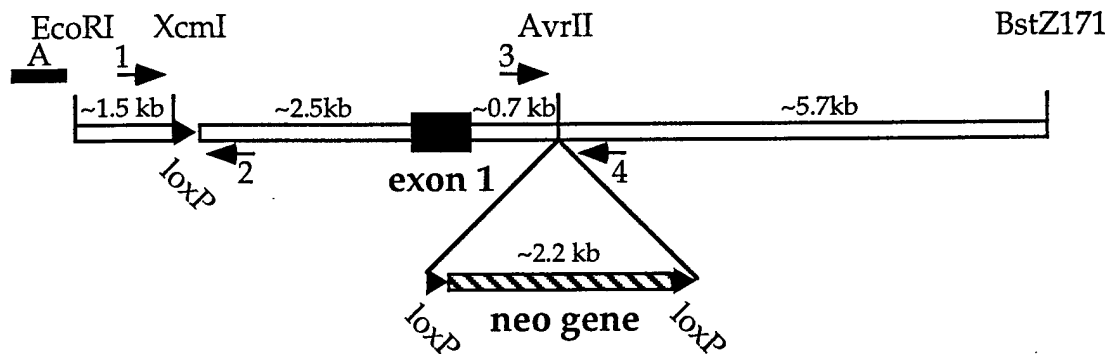


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## Appendices



**Figure 1. EGF receptor gene targeting construct .**

**A** is the probe used for the Southern bolt analysis to determine the correct recombination, which is outside the 5'-end of the targeting vector.

**1** and **2** are the oligo primers used to determine the mutant allele.

**3** and **4** are the oligo primers used to determine the deletion of **neo** gene by EIIa-Cre mice.

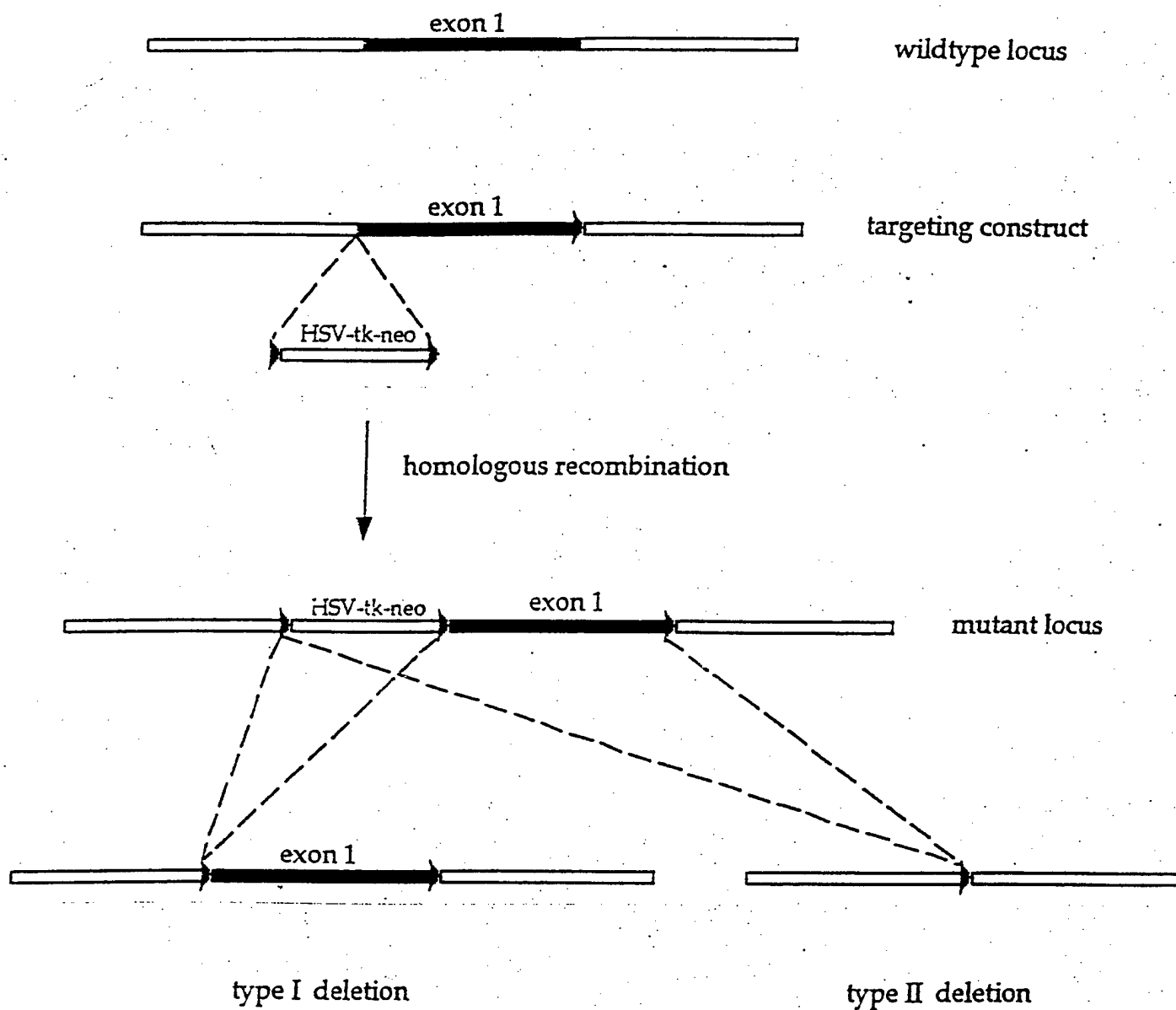
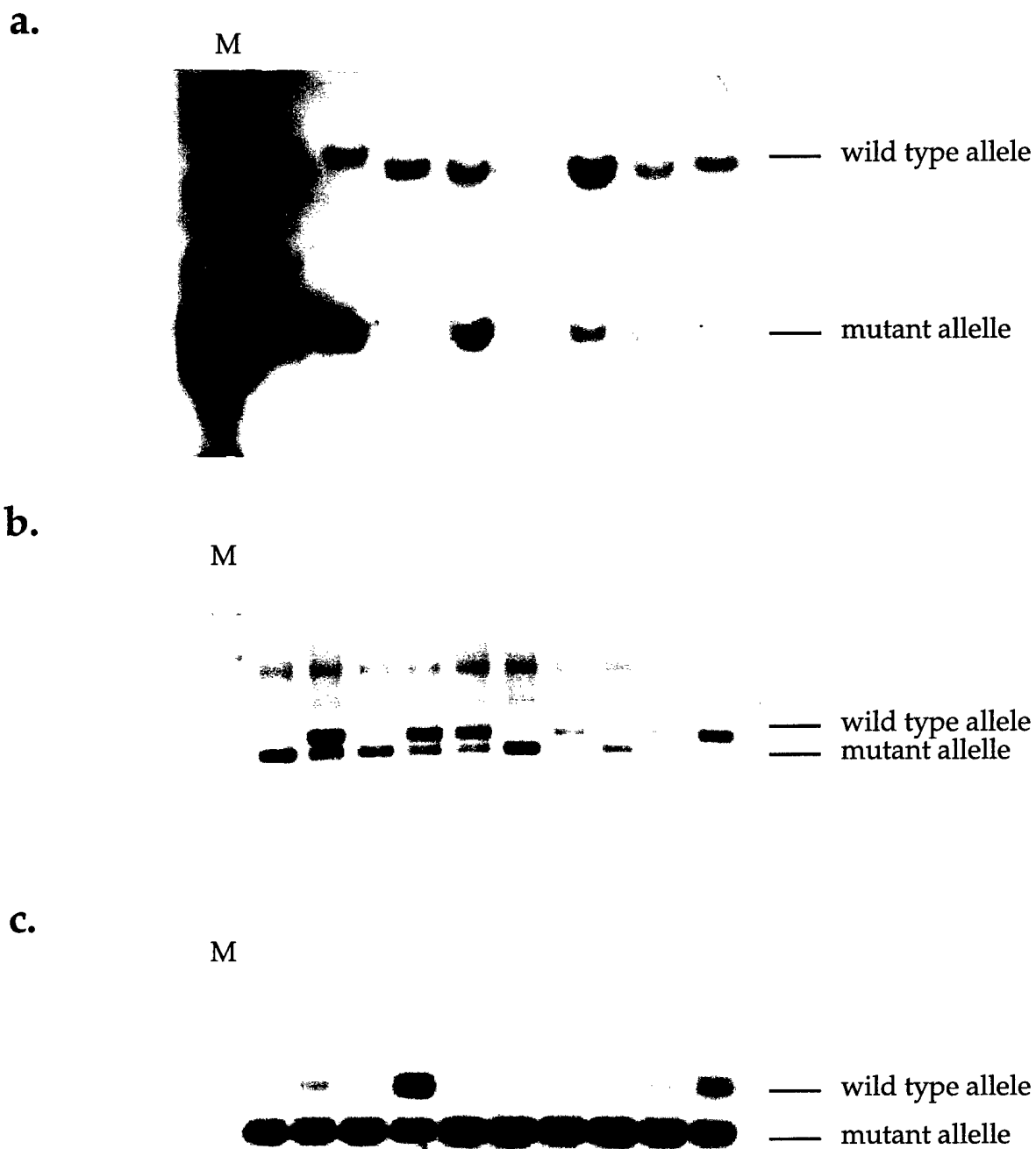


Figure 2. Strategy to generate genes flanking by loxP sites in ES cells. Genetic locus, targeting construct and mutant locus as well as two types of cre-mediated deletions are shown. The loxP site:  $\blacktriangleright$ .



**Figure 3. Southern Blot analysis and PCR analysis for mutant allele of EGF receptor gene.**

a. using a DNA probe 5'- end outside of the targeting vector

b. using oligo primers 1 and 2 (see figure 1)

c. using oligo primers 3 and 4 ( see figure 1)

M is DNA marker